95974

XYLANASES WITH ENHANCED THERMOPHILICITY AND ALKALOPHILICITY

Inventor

Wing L Sung

Applicant

National Research Council

XYLANASES WITH ENHANCED THERMOPHILICITY AND ALKALOPHILICITY

The present invention relates to xylanases. More specifically, the invention relates to xylanases, and modified xylanases with improved performance at conditions of high temperature and pH.

BACKGROUND OF THE INVENTION

Xylanases are a group of enzymes with wide commercial utility. A major application of xylanases is for pulp biobleaching in the production of paper. In addition, xylanases have been used as clarifying agents in juices and wines, as enzymatic agents in the washing of precision devices and semiconductors (e.g. U.S. Pat. No. 5,078,802), and they are also used for improving digestibility of poultry and swine feed.

In the manufacturing of pulp for the production of paper, fibrous material is subjected to high temperatures and pressures in the presence of chemicals. This treatment converts the fibers to pulp and is known as pulping. Following pulping, the pulp is bleached. Xylanase enzymes are used to enhance the bleaching of the pulp. The xylanase treatment allows subsequent bleaching chemicals such as chlorine, chlorine dioxide, hydrogen peroxide, or combinations of these chemicals to bleach pulp more efficiently. Pretreatment of pulp with xylanase increases the whiteness and quality of the final paper product and reduces the amount of chlorine-based chemicals which must be used to bleach the pulp. This in turn decreases the chlorinated effluent produced by such processes.

The most important chemical pulping process is kraft pulp. For kraft pulp, following pulping, and prior to the treatment of pulp with xylanase, the pulp is at about a temperature of 55-70°C and at a highly alkaline pH (e.g. Nissen et al., 1992). A drawback of many commercially available wild-type xylanases, is that these enzymes exhibit an acidic pH optimum and a temperature optimum of about 55°C. Therefore, in order to effectively utilize xylanases for bleaching applications, the pulp must be acidified to a pH approximating the optimal pH for the specific xylanase used. In addition, the hot pulp must be cooled to a temperature close to the optimal temperature for enzymatic activity of the selected xylanase. Decreasing pulp temperatures for xylanase treatment decreases the efficiency of the subsequent chemical bleaching. Acidification of pulp requires the use of large quantities of acids. Further, the addition of acids leads to corrosion, which lessens the lifetime of process equipment. Thus,

xylanases optimally active at temperatures and pH conditions approximating the conditions of the pulp would be useful and beneficial in pulp manufacturing.

Xylanases which exhibit greater activity at higher temperatures could be used to treat pulp immediately following the pulping process, without the need to cool the pulp. Similarly, xylanases which exhibit greater activity at higher pH conditions would require less or no acid to neutralize the pulp. The isolation of, or the genetic manipulation of, xylanases with such properties would provide several advantages and substantial economic benefits within a variety of industrial processes.

Several approaches directed towards improving xylanase for use in pulp-bleaching within the prior art include the isolation of thermostable xylanases from extreme thermophiles that grow at 80-100°C, such as *Caldocellum saccharolyticum*, *Thermatoga maritima* and *Thermatoga sp.* Strain FJSS-B.1 (Lüthi et al. 1990; Winterhalter et al. 1995; Simpson et al. 1991). However, these thermostable xylanase enzymes are large, with molecular masses ranging from 35-120 kDa (320-1100 residues), and exhibit a reduced ability to penetrate the pulp mass compared with other smaller xylanases which exhibit better accessibility to pulp fibers. In addition, some of the extremely thermophilic xylanases, such as *Caldocellum saccharolyticum* xylanase A, exhibit both xylanase and cellulase activities (Lüthi et al. 1990). This additional cellulolytic activity is undesirable for pulp bleaching, due to its detrimental effect on cellulose, the bulk material in paper. Furthermore, hyper-thermostable xylanase enzymes which function normally at extremely high temperatures have low specific activities at temperatures in the range for optimal pulp bleaching (Simpson et al. 1991).

A number of xylanases have been modified by protein engineering to improve their properties for industrial applications. For instance, U.S. 5,759,840 (Sung et al.), and U.S. 5,866,408 (Sung et al.) disclose mutations in the N-terminal region (residues 1-29) of *Trichoderma reesei* xylanase II (TrX). Three mutations, at residues 10, 27 and 29 of TrX, were found to increase the enzymatic activity of the xylanase enzyme at elevated temperatures and alkaline pH conditions.

U.S. 5,405,769 (Campbell et al.), discloses modification of *Bacillus circulans* xylanase (BcX) using site-directed mutagenesis to improve the thermostability of the enzyme. The site specific mutations include replacing two amino acids with Cys residues to create intramolecular disulfide bonds. In addition, specific residues in the N-terminus of the enzyme were mutated which were also found to further improve the thermostability of the enzyme. In *in vitro* assays,

the disulfide mutants showed thermostability at 62° C, an improvement of 7° C over the native BcX xylanase enzyme. However, these thermostable disulfide mutants showed no gain in thermophilicity in laboratory assays in subsequent studies (Wakarchuck et al., 1994). Mutations T3G (i.e. threonine at position 3 replaced with Gly; BcX xylanase amino acid numbering), D4Y(F) and N8Y(F) near the N-terminus of the BcX xylanase enzyme provided thermostability to 57° C, an increase of 2° C over the native BcX (U.S. 5,405,769). However, the use of these enzymes within industrial applications still requires cooling and acidification of pulp following pretreatment, prior to enzyme addition. Therefore, further increases in thermostability, thermophilicity and pH optima are still required.

Turunen et al. (2001) discloses mutations (N11D, N38E, Q162H) of TrX II at positions 11, 38 and 162, complement similar disulfide bond (S110C/N154C) to improve the thermostability of the xylanase. However, these mutations including N11D also have an adverse effect on both the thermophilicity and the alkalophilicity of the xylanase, resulting in a decrease of enzymatic activity at higher temperatures and the neutral-alkaline pH, as compared to native TrX II.

There is a need in the prior art to obtain novel xylanases which exhibit increased enzymatic activity at elevated temperatures and pH conditions, suitable for industrial use. It is an object of the invention to overcome drawbacks in the prior art.

The above object is met by the combination of features of the main claim, the sub-claims disclose further advantageous embodiments of the invention.

SUMMARY OF THE INVENTION

The presènt invention relates to xylanases. More specifically, the invention relates to xylanses, and modified xylanases with improved performance at conditions of high temperature and pH.

This invention relates to a xylanase comprising at least one substituted amino acid residue at a position selected from the group consisting of amino acid 11, 116, 118, 144, and 161, with the position determined from sequence alignment of the modified xylanase with *Trichoderma reesei* xylanase II amino acid sequence defined in SEQ ID NO:16. Preferably, the xylanase exhibits improved thermophilicity, alkalophilicity, broader effective pH range, expression efficiency or a combination thereof, in comparison to a corresponding native TrX xylanase.

The present invention also provides for the xylanase as defined above wherein the xylanase is a modified xylanase and at least one substituted amino acid residue is at position 116. Preferably the substituted amino acid is Gly.

The present invention also embraces the xylanase, modified at position 116 as defined above and further comprising a His at positions 10 and 105, a Met at position 27, a Leu at position 29, an Ala at positions 75 and 125, a Glu at position 129 and a Arg at position 144.

This invention includes the xylanase modified at position 116 as defined above and further comprising a His at positions 10 and 105, a Met at position 27, a Leu at position 29, an Ala at positions 75 and 125, a Glu at position 129 and a Arg at position 144.

This invention describes the xylanase modified at position 116 as defined above and further comprising a His at positions 10 and 105, an Asp at position 11, a Met at position 27, a Leu at position 29, an Ala at positions 75 and 125, a Glu at position 129 and a Arg at positions 144 and 161.

The present invention also provides for the modified xylanase as defined above wherein the at least one substituted amino acid residue is at position 144. Preferably the substituted amino acid is Arg.

The present invention embraces the xylanase modified at position 144 as defined above and further comprising a His at positions 10 and 105, a Met at position 27, a Leu at position 29, an Ala at positions 75 and 125 and a Glu at position 129.

The present invention also provides for the modified xylanase as defined above wherein the at least one substituted amino acid residue is at position 161. Preferably the substituted amino acid is Arg.

This invention embraces the xylanase modified at position 161 as defined above and further comprising a His at positions 10 and 105, a Met at position 27, a Leu at position 29, an Ala at positions 75 and 125, a Glu at position 129 and an Arg at postion 144.

The present invention also provides for the modified xylanase as defined above wherein the at least one substituted amino acid residue is at position 11. Preferably the substituted amino acid is Asp.

This invention embraces the xylanase modified at position 11 as defined above and further comprising a His at positions 10 and 105, a Met at position 27, a Leu at position 29, an Ala at positions 75 and 125, a Glu at position 129 and an Arg at postions 144 and 161.

The present invention also provides for the modified xylanase as defined above wherein the at least one substituted amino acid residue is at position 118. Preferably the substituted amino acid is Cys.

The present invention also embraces the xylanase modified at position 118 as defined above and further comprising a His at positions 10 and 105, a Met at position 27, a Leu at position 29, an Ala at positions 75 and 125, a Glu at position 129 and a Arg at position 144.

This invention includes the xylanase modified at position 118 as defined above and further comprising a His at positions 10 and 105, a Met at position 27, a Leu at position 29, an Ala at positions 75 and 125, a Glu at position 129 and a Arg at position 144.

This invention describes the xylanase modified at position 118 as defined above and further comprising a His at positions 10 and 105, an Asp at position 11, a Met at position 27, a Leu at position 29, an Ala at positions 75 and 125, a Glu at position 129 and a Arg at positions 144 and 161.

The present invention is also directed to the modified xylanases, as defined above, wherein the modified xylanases are derived from a Family 11 xylanase, preferably a *Trichoderma reesei* xylanase.

The present invention pertains to a modified xylanase comprising at least one substituted amino acid residue, wherein the modified xylanase is characterized as having a maximum effective temperature (MET) between about 69°C to about 84°C, and wherein the modified xylanase is a Family 11 xylanase obtained from a *Trichoderma* sp. Preferably, the MET is between about 70° to about 80°C

This invention also includes a modified xylanase comprising at least one substituted amino acid residue, wherein the modified xylanase is characterized as having a maximum effective pH (MEP) between about pH 5.8 to about pH 8.4, and wherein the modified xylanase is a Family 11 xylanase obtained from a *Trichoderma* sp. Preferably, the MEP is between about pH 6.0 to about pH 8.0.

The present invention is directed to a modified xylanase comprising at least one substituted amino acid residue, wherein the modified xylanase is characterized as having a maximum effective temperature (MET) between about 69°C to about 84°C, and a maximum effective pH (MEP) between about pH 5.8 to about pH 8.4. Prefereably, the MET is between about 70° to about 80°C, and the MEP is between about pH 6.0 to about pH 8.0.

The present invention also relates to a modified xylanase selected from the group consisting of:

TrX-HML-75A105H-125A129E-144R;
TrX-HML-75A105H-125A129E-144R161R;
TrX-116G;
TrX-118C;
TrX-HML-75A105H-116G-125A129E-144R;
TrX-HML-75A105H-118C-125A129E-144R;
TrX-H-11D-ML-75A105H-125A129E-144R161R;
TrX-H-11D-ML-75A105H-116G-125A129E-144R161R;
TrX-H-11D-ML-75A105H-118C-125A129E-144R161R; and
TrX-H-11D-ML-75A105H-116G118C-125A129E-144R161R;

According to the present invention, there is also provided a modified xylanase comprising at least one substituted amino acid residue, and characterized as having a maximum effective temperature (MET) between about 69°C to about 84°C, wherein the modified xylanase is a Family 11 xylanase obtained from a *Trichoderma* sp. Furthermore the present invention relates to a modified Family 11 xylanase obtained from a *Trichoderma* sp. characterized as having a MET between about 70° to about 80°C. The present invention also includes the modified Family 11 xylanase obtained from a *Trichoderma* sp. characterized as having a MET between about 69°C to about 84°C and a maximum effective pH (MEP) between about 5.8 to about 8.4. This invention also pertains to the modified xylanase as just defined, wherein the MEP is between about 6.0 to about 8.0.

The present invention is directed to the use of the modified xylanase as defined above in an industrial process. Also included is an industrial process, wherein the industrial process comprises bleaching of pulp, processing of precision devices, or improving digestibility of poultry and swine feed.

This summary of the invention does not necessarily describe all necessary features of the invention but that the invention may also reside in a subcombination of the described features.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features of the invention will become more apparent from the following description in which reference is made to the appended drawings wherein:

FIGURE 1 shows an amino acid sequence alignment among Family 11 xylanases. The amino acid numbering is compared with *Trichoderma reesei* xylanase II (Tr2) as indicated at the top of the sequences. The residues at position 75 and 105 (relative to Tr2) are in italic and indicated with an asterisk. The amino acids common to at least 75% of the listed Family 11 xylanases are indicated in bold. The residues common to all Family 11 xylanases are underlined. For xylanases with a cellulose-binding domain, only the catalytic core sequences are presented.

FIGURE 2 shows the nucleotide sequence of TrX xylanase (SEQ ID NO:39), and the synthetic oligonucleotides used to construct the sequence encoding the *Trichoderma reesei* xylanase II enzyme (TrX) in the plasmid pTrX.

FIGURE 3 shows the effect of temperature on the enzymatic activity of modified xylanase TrX-HML-75A105H-125A129E-144R, compared with TrX- HML-75A105H-125A129E, at pH 5.5 during 30-min incubations. The data are normalized to the activity observed at 40°C.

FIGURE 4 shows the effect of temperature on the enzymatic activity of modified xylanases TrX-116G and TrX-118C, compared to native TrX, at pH 5.0 during 30-min incubations. The data are normalized to the activity observed at 40° C.

FIGURE 5 shows the effect of temperature on the enzymatic activity of modified xylanases TrX-HML-75A105H-116G-125A129E-144R and TrX-H-11D-ML-75A105H-116G-125A129E-144R161R, as compared to native TrX, TrX-HML, TrX-HML-75A105H-125A129E and TrX-HML-75A105H-125A129E-144R, at pH 5.5 during 30-min incubations. The data are normalized to the activity observed at 40° C.

FIGURE 6 shows the effect of temperature on the enzymatic activity of modified xylanases TrX-H-11D-ML-75A105H-116G118C-125A129E-144R161R, TrX-HML-75A105H-116G-125A129E-144R, TrX-H-11D-ML-75A105H-116G-125A129E-144R161R, TrX-

HML-75A105H-118C-125A129E-144R and TrX-H-11D-ML-75A105H-118C-125A129E-144R161R, as compared to TrX-HML-75A105H-125A129E-144R, at pH 6.0 during 30-min incubations. The data are normalized to the activity observed at 40° C.

FIGURE 7 shows the effect of temperature on the enzymatic activity of modified xylanases TrX-HML-75A105H-116G-125A129E-144R and TrX-H-11D-ML-75A105H-116G-125A129E-144R161R, TrX-HML-75A105H-118C-125A129E-144R and TrX-H-11D-ML-75A105H-118C-125A129E-144R161R, as compared to TrX-HML-75A105H-125A129E-144R, at pH 6.0 during 30-min incubations. The data are normalized to maximum activity for each enzyme.

FIGURE 8 shows the effect of temperature on the enzymatic activity of modified xylanases TrX-H-11D-ML-75A105H-125A129E-144R161R, as compared to TrX-HML-75A105H-125A129E-144R161R, at pH 6.0 during 30-min incubations. The data are normalized to the activity observed at 40° C.

FIGURE 9 shows the pH /activity profile of modified xylanase enzymes TrX-H-11D-ML-75A105H-116G118C-125A129E-144R161R, TrX-H-11D-ML-75A105H-116G-125A129E-144R161R, TrX-HML-75A105H-116G-125A129E-144R and TrX-HML-75A105H-118C-125A129E-144R, as compared to TrX-HML-75A105H-125A129E-144R161R and TrX-HML-75A105H-125A129E, over pH 5.0-8.0 at 65° C during 30-min incubation. The data are normalized to the pH exhibiting optimal activity for each enzyme.

FIGURE 10 shows the pH /activity profiles of modified xylanases TrX-116G and TrX-118C, as compared to native TrX, over pH 4.5-7.0 at 50° C during 30-min incubation. The data are normalized to the pH exhibiting optimal activity for each enzyme.

FIGURE 11 shows the maximum effective temperature (MET) and maximum effective pH (MEP) values of several of the modified enzymes of the present invention. The MET and MEP are the highest temperature and pH, respectively, at which a xylanase exhibits at least 80% of its optimal activity (using soluble birchwood xylan as a substrate; see method for complete details of assays). These data points were obtained from the data presented in Figures 3 to 10.

DESCRIPTION OF PREFERRED EMBODIMENT

The present invention relates to xylanases. More specifically, the invention relates to xylanase andmodified xylanases with improved performance at conditions of high temperature and pH.

The following description is of a preferred embodiment by way of example only and without limitation to the combination of features necessary for carrying the invention into effect.

The mechanism by which xylanases facilitate bleaching of pulp is not fully understood. It has been postulated that the coloured lignin is connected to crystalline cellulose through xylan and xylanase enzymes facilitate bleaching of pulp by hydrolysing xylan, releasing coloured lignin in the pulp. Xylanases and modified xylanases, as outlined herein, may be used for the purposes of bleaching pulp or other applications requiring activities at temperatures and pH above that of the wild-type enzyme. For the biobleaching of pulp, the preferred xylanase is derived from a xylanase classified in Family 11 (see Table 1), however, the modifications disclosed herein need not be limited to only Family 11 xylanases and may include other xylanase enzymes. Furthermore, the modifications as outlined herein may be found in native xylanase proteins, and these native xylanase enzymes may exhibit the desired features as described herein, and are included within the present invention.

Family 11 xylanase enzymes are a group of small enzymes of relatively low molecular mass (approximately 20 kDa, and about 200 amino acid residues. The small size associated with Family 11 xylanases permits ready penetration of the pulp mass. Furthermore, Family 11 xylanases are free of cellulase activity.

One aspect of the present invention is directed to a modified Family 11 xylanase obtained from a *Trichoderma* sp. comprising at least one substituted amino acid residue, and characterized as having a maximum effective temperature (MET; see definition below) between about 69°C to about 84°C. Preferably, the modified xylanase is characterized as having a MET between about 70° to about 80°C. This invention also includes a modified xylanase comprising at least one

substituted amino acid residue, and is characterized as having a maximum effective pH (MEP; see definition below) between about 5.8 to about 8.4. Preferably, the MEP is between about 6.0 to about 8.0.

This invention also pertains to a modified xylanase obtained from *Trichoderma*, comprising at least one substituted amino acid, and characterized as having a maximum effective temperature (MET) between about 69°C to about 84°C, and a maximum effective pH (MEP) between about 5.8 to about 8.4. Preferably the MET is between about 70°to about 80°C, and the MEP is between about 6.0 to about 8.0.

This invention also pertains to a native family 11 xylanase characterized as having a maximum effective temperature (MET) between about 69°C to about 84°C, and a maximum effective pH (MEP) between about 5.8 to about 8.4. Preferably the MET is between about 70°to about 80°C, and the MEP is between about 6.0 to about 8.0.

By "maximum effective temperature" or "MET" it is meant the highest temperature at which a xylanase exhibits at least 80% of its optimal activity. This test is typically carried out using soluble birchwood xylan as a substrate at pH 5.5 or 6.0, and for a 30 min period. Results from assays used to characterize modified xylanases are presented in Figures 3 to 8 and involved a 30-min incubation at pH 5.5 or 6.0. A summary of the MET of several enzymes of the present invention, determined from Figures 3 to 8 is presented in Figure 11. Experiments demonstrate that the MET of a xylanase differs on different substrates. Therefore, it is to be understood that with different substrates, different MET values will be obtained (data not presented). For the purposes of evaluating xylanases of the present invention, the soluble birchwood xylan substrate is used (see example 3).

By "maximum effective pH" or "MEP" it is meant the highest pH at which a xylanase exhibits at least 80% of its optimal activity. This test is carried out using soluble birchwood xylan as a substrate, at 65°C, and for a 30-min period. Results from assays used to characterize modified xylanases are presented in Figures 9 and 10 and involved a 30-min incubation at 65°C. A summary of the MEP of several enzymes of the present invention is presented in Figure 11.

Experiments demonstrate that the MEP of a xylanase differs on different substrates. For example, on kraft pulp prepared from soft wood or hardwood, a MEP of 9.2 has been observed (data not presented). Therefore, it is to be understood that with different substrates, different MEP values will be obtained. For the purposes of evaluating xylanases of the present invention, the soluble birchwood xylan substrate is used (see example 4).

TABLE 1. Family 11 xylanase enzymes

Microbe	Xylanase	SEQ ID NO
Aspergillus niger	Xyn A	SEQ ID NO: 1
Aspargillus awamori var.kawachi	Xyn B	SEQ ID NO: 19
Aspergillus kawachii	Xyn C	--
Aspergillus tubigensis	Xyn A	SEQ ID NO: 2
Bacillus circulans	Xyn A	SEQ ID NO: 3
Bacillus pumilus	Xyn A	SEQ ID NO: 4
Bacillus subtilis	Xyn A	SEQ ID NO: 5
Cellulomonas fimi	Xyn D	--
Chainia spp.	Xyn	--
Clostridium acetobutylicum	Xyn B	SEQ ID NO: 6
Clostridium stercorarium	Xyn A	SEQ ID NO: 7
Fibrobacter succinognees	Xyn II	SEQ ID NO: 18
Neocallimasterix patriciarum	Xyn A	--
Nocardiopsis dassonvillei	Xyn II	--
Ruminococcus flavefaciens	Xyn A	SEQ ID NO: 8
Schizophyllum cimmune	Xyn	SEQ ID NO: 9
Streptomyces lividans	Xyn B	SEQ ID NO: 10
Streptomyces lividans	Xyn C	SEQ ID NO: 11
Streptomyces sp. No. 36a	Xyn	SEQ ID NO: 12
Streptomyces thermoviolaceus	Xyn II	--
Thermomonospora fusca	Xyn A	SEQ ID NO: 13
Thermomyces lanuginosus	Xyn	SEQ ID NO: 20
Trichoderma harzianum	Xyn	SEQ ID NO: 14
Trichoderma reesei	Xyn I	SEQ ID NO: 15
Trichoderma reesei	Xyn II	SEQ ID NO: 16
Trichoderma viride	Xyn	SEQ ID NO: 17

Family 11 xylanases share extensive amino acid sequence similarity (Figure 1). Structural studies of several Family 11 xylanases indicate that Family 11 xylanases from bacterial and fungal origins share the same general molecular structure (U.S. 5,405,769; Arase et al 1993). In addition, most Family 11 xylanases identified so far exhibit three types of secondary structure, including beta-sheets, turns and a single alpha helix. The helix of *Trichoderma reesei* xylanase II enzyme encompasses the region from amino acid 151 to amino acid 162 (Torronen et. al. 1995).

A xylanase is classified as a Family 11 xylanase if it comprises amino acids common to other Family 11 xylanases, including two glutamic acid (E) residues which may serve as catalytic residues. The glutamic acid residues are found at positions 86 and 177 (see Figure 1; based on Tr2 (*Trichoderma reesei* xylanase II enzyme) amino acid numbering).

Most of the Family 11 xylanases identified thus far are mesophilic and have low-molecular masses (20 kDa). However, this family also includes at least two thermostable xylanases of higher molecular mass, *Thermomonospora fusca* xylanase A (TfX-A) of 296 amino acids and a molecular mass of approximately 32 kDa (Irwin et. al., 1994); Wilson et al. 1994, WO 95/12668) and *Clostridium stercorarium* xylanase A of 511 amino acids and a molecular mass of approximately 56 Kda. The *Clostridium stercorarium* xylanase A enzyme exhibits maximum activity at a temperature of 70° C (Sakka et al.,1993).

The large thermostable Family 11 xylanases differ from the small mesophilic enzymes by the possession of a hydrophobic cellulose-binding domain (CBD) in the extended C-terminus of the enzyme. The TfX-A enzyme is composed of a catalytic core sequence of 189 residues common to all Family 11 xylanases, and a cellulose binding domain of 107 residues. The larger *C. stercorarium* xylanase A has 2 copies of the cellulose binding domain.

Site-directed mutagenesis has been used in the present invention to produce mutations in xylanases which render the enzyme more thermophilic and alkalophilic compared to the native enzyme. Preferably, the mutant xylanase is one derived from a Family 11 xylanase. More preferably, the mutant xylanase of the present invention comprises a mutant *Trichoderma reesei* xylanase II enzyme.

Therefore, it is considered within the scope of the present invention that xylanases, including Family 11 xylanases for example but not limited to *Trichoderma reesei* xylanase II, *Trichoderma reesei* xylanase I, *Trichoderma viride* xylanase, *Streptomyces lividans* xylanase B

and *Streptomyces lividans* xylanase C, may be modified following the general approach and methodology as outlined herein. It is also considered within the scope of the present invention that non-Family 11 xylanases may also be modified following the general principles as described herein in order to obtain a xylanase enzyme that exhibits thermophilicity and alkalophilicity.

By the term "thermophilicity" it is meant that an enzyme is active, or more active, at a higher temperature when compared with the activity of another enzyme when all other conditions remain constant. For example, xylanase 1 exhibits increased thermophilicity compared to xylanase 2 if xylanase 1 is capable of, or is more active in, hydrolysing xylan at a higher temperature than xylanase 2, under identical conditions using the same substrate. As most xylanases are effective at a higher temperature when hydrolysing pure xylan rather than pulp, comparative analysis should be made using the same substrate. Quantitative measures of thermophilicity referred to herein use pure xylan substrates unless otherwise indicated.

By "thermostability" it is meant the ability of an enzyme to be stored or incubated at high temperature conditions, typically in the absence of substrate, and then exhibit activity when returned to standard assay conditions. For example, xylanase 1 is said to display increased thermostability compared to xylanase 2 if xylanase 1 retains a greater amount of activity than xylanase 2 after being maintained at a certain temperature (typically a higher temperature), for example but not limited to, 70° C for 24 hours, followed by assay at a lower temperature. In contrast to thermophilicity, thermostability relates to the remaining enzyme activity following an incubation in the absence of substrate.

These use of these two terms (thermophilicity and thermostability) has been confused within the prior art as they have been used interchangeably. However, the use of the terms as defined herein is consistent with the usage of the terms in the art (Mathrani and Ahring, 1992).

By "alkalophilicity" it is meant that an enzyme is active, or more active, at a higher pH when compared with the activity of another enzyme when all other conditions remain constant. For example, xylanase 1 exhibits increased alkalophilicity compared to xylanase 2 if xylanase 1 is capable of hydrolysing xylan at a higher pH than xylanase 2. Typically alkalophilicity relates to enzyme activity in the presence of xylan substrate.

By "broader range of effective pH", it is meant that an enzyme is active, or more active, at a higher ph, a lower pH, or both a higher and lower pH, when compared to the activity of another enzyme when all other conditions remain constant. For example, which is not to be

considered limiting, xylanase 1 exhibits broader range of effective pH compared to xylanase 2, if xylanase 1 is capable of hydrolysing xylan over a pH of 5.5 – 8.0 at close to optimal (80%) activity, while xylanase 2 can only maintain 80% optimal activity at a narrower range of pH 5.5 – 7.5.

By "TrX numbering" it is meant the numbering associated with the position of amino acids based on the amino acid sequence of TrX (Xyn II - Table 1; Tr2 - Figure 1; SEQ ID NO:16). As disclosed below and as is evident upon review of Figure 1, Family 11 xylanases exhibit a substantial degree of sequence similarity. Therefore, by aligning the amino acids to optimize the sequence similarity between xylanase enzymes and by using the amino acid numbering of TrX as the basis for numbering, the positions of amino acids within other xylanase enzymes can be determined relative to TrX.

By "expression efficiency", it is meant that the suitability or ease of active enzyme or enzymatic activity to be produced by the production host, and is typically calculated as quantity of active enzyme or enzymatic activity generated per unit volume of the fermentation culture when all fermentation conditions remain constant. For example, which is not to be considered limiting, xylanase 1 has improved expression efficiency compared to xylanase 2 if xylanase is produced 3-fold as much as xylanase 2 in a unit volume of culture by the same host. A non-limiting example of such a host is *E. coli*.

By modified xylanase, it is meant the alteration of a xylanase molecule using techniques that are known to one of skill in the art. These techniques include, but are not limited to, site directed mutagenesis, cassette mutagenesis, random mutagenesis, synthetic oligonucleotide construction, cloning and other genetic engineering techniques.

As described in more detail herein, several mutant xylanases have been prepared that exhibit increased thermophilicity, alkalophilicity and thermostability when compared to native xylanase. A list of several of mutants, which is not to be considered limiting in any manner, is presented in Table 2.

Furthermore, the present is directed to a modified Family 11 xylanase, for example but not limited to a xylanase obtained from a *Trichoderma* sp., that comprises at least one substituted amino acid residue, and characterized as having a maximum effective temperature (MET) between about 69°C to about 84°C. Preferably, the modified xylanase is characterized as having a MET between about 70° to about 80°C. This invention also pertains to a modified xylanase,

for example but nto limited to a xylanase obtained from *Trichoderma*, comprising at least one substituted amino acid, and characterized as having a maximum effective pH (MEP) between about 5.8 to about 8.4. Preferably the MEP is between about 6.0 to about 8.0. This invention also pertains to a modified xylanase, for example but not limited to a xylanase obtained from *Trichoderma*, comprising at least one substituted amino acid, and characterized as having a maximum effective temperature (MET) between about 69°C to about 84°C, and a maximum effective pH (MEP) is between about 5.8 to about 8.4. Preferably the MET is between about 70°to about 84°C, and the MEP is between about 6.0 to about 8.0.

Furthermore, the present invention also relates to a native family 11 xylanase characterized as having a maximum effective temperature (MET) between about 69°C to about 84°C, and a maximum effective pH (MEP) between about 5.8 to about 8.4. Preferably the MET is between about 70°to about 80°C, and the MEP is between about 6.0 to about 8.0.

Determination of the MET and MEP of a xylanase may be carried out as follows:

i) measure the temperature profile of a xylanase as outlined in Example 3. The temperatures for which at least 80% of the optimal (maximum) activity are determined, and the highest temperature is the MET;

ii) measure the pH profile of a xylanase as outlined in Example 4. The pH for which at least 80% of the optimal (maximum) activity is determined, and the highest pH is the MEP.

These values may then be plotted as shown in Figure 11.

Table 2: Modified xylanases

Xylanase	Description
TrX-HML	TrX with N10H, Y27M, and N29L (see U.S. 5,759,840)
TrX-HML-105R	TrX N10H, Y27M, N29L and L105R
TrX-HML-75A-105R	TrX N10H, Y27M, N29L, S75A and L105R
TrX-HML-75G-105R	TrX N10H, Y27M, N29L, S75G and L105R
TrX-HML- GRAE	TrX N10H, Y27M, N29L, S75G, L105R, Q125A and I129E
TrX-HML- AHAE	TrX N10H, Y27M, N29L, S75A, L105H, Q125A and I129E
TrX-HML- AHAE-R	TrX N10H, Y27M, N29L, S75A, L105H, Q125A, I129E and 144R
TrX-HML- AHAE-RR	TrX N10H, Y27M, N29L, S75A, L105H, Q125A, I129E, 144R, and Q161R
TrX-116G	TrX D116G
TrX-118C	TrX Y118C
TrX-HML-AHGAE-R	TrX N10H, Y27M, N29L, S75A, L105H, D116G, Q125A, I129E and H144R
TrX-HML-AHCAE-R	TrX N10H, Y27M, N29L, S75A, L105H, Y118C, Q125A, I129E and H144R
TrX-H-11D-ML-AHAE-RR	TrX N10H, N11D, Y27M, N29L, S75A, L105H, Q125A and I129E, H144R and Q161R
TrX-H-11D-ML-AHGAE-RR	TrX N10H, N11D, Y27M, N29L, S75A, L105H, D116G, Q125A, I129E, H144R and Q161R
TrX-H-11D-ML-AHCAE-RR	TrX N10H, N11D, Y27M, N29L, S75A, L105H, Y118C, Q125A, I129E, H144R and Q161R
TrX-H-11D-ML-AHCAE-RR	TrX N10H, N11D, Y27M, N29L, S75A, L105H, D116G, Y118C, Q125A, I129E, H144R and Q161R

Substitution at position 11, 116, 118, 144 or 161 does not significantly change the specific activity of the xylanase enzyme compared to that of native xylanase (see Table 4, Example 2-3).

Improving the Expression Efficiency of Xylanase

The mutant xylanases
TrX-H-**11D**-ML-75A105H-125A129E-144R161R, (TrX H 11D-ML-AHAE-RR);
TrX-H-**11D**-ML-75A105H-116G-125A129E-144R161R (TrX H11D-ML-AHGAE-RR); and
TrX-H-**11D**-ML-75A105H-118C-125A129E-144R161R (TrX H11D-ML-AHCAE-RR),
all bearing the mutation of N11D, have consistently been expressed and produce from about 2.5 top about 4.3 fold as much protein than their precursors without this mutation (see Table 5, Example 2-4). These results suggested that this mutation improves the yield of the production of xylanases, an important factor in any production in industrial scale. This improvement in expression efficiency of xylanase is achieved without any decrease of thermophilicity and alkalophilicity of the xylanase.

Increasing the Thermophilicity of Xylanase

The mutation of position 144 to Arg has improved the enzymatic activity of mutant xylanase TrX-HML-75A105H-125A129E-**144R** (TrX-HML-AHAE-R) in the hydrolysis of xylan at higher temperatures (Figure 3), when compared to the precursor xylanase TrX-HML-75A105H-125A129E that lacks this mutation. Therefore, the present invention provides a native or a modified xylanase comprising a basic amino acid, for example but not limited to Arg, at position 144. Preferably the native or modified xylanase with the basic amino acid at position 144 exhibits a MET between about 69°C to about 84°C.

Two mutations at positions 116 and 118 to Gly and Cys, respectively, also demonstrate improved activity of xylanase at high temperatures. Compared to native TrX, the single point mutants TrX-**116G** and TrX-**118C** exhibit greater activity at higher temperatures (Figure 4), with a temperature optimum at 55°C, v. 50°C exhibited by native TrX.

The same enhancement in thermophilicity by these two mutations (116 and 118 to Gly and Cys, respectively) is also observed in:
TrX-HML-75A105H-**116G**-125A129E-144R (TrX-HML-AHGAE-R); and
TrX-HML-75A105H-**118C**-125A129E-144R (TrX-HML-AHCAE-R),
when compared to the precursor xylanase, TrX-HML-75A105H-125A129E-144R (TrX-HML-AHAE-R) at pH 5.5 (see Figure 5, 116G mutant) and pH 6.0 (Figures 6 and 7).

The improvement in thermophilicity by the mutations at position 116 to a small non-polar residue is unexpected as a majority of the natural xylanases including the thermophilic xylanases (for example, Tf, Tl, Cs, Figure 1) possess negatively charged amino acids, aspartic acid (D, 66%, Figure 1) and glutamic acid (E, 10%, Figure 1), or a polar, uncharged amino acid glutamine (Q, 15%, Figure 1) at this position. No known xylanases possess a Gly at position 116. Therefore, the present invention also pertains to a native or a modified xylanase comprising a non-polar amino acid, for example but not limited to Gly, at position 116. Preferably the native or modified xylanase with the non-polar amino acid at position 116 exhibits a MET between about 69°C to about 84°C.

The improvement of thermophilicity based on the mutation at position 118 to cysteine is also unexpected, as most xylanases including the thermophilic xylanases (Tf, Tl, Cs, Figure 1) possess a tyrosine (Y, 60%, Figure 1) and trytophan (W, 10%, Figure 1). The only xylanases possessing Cysteine at position 118 are among the mesophilic *Aspergillus niger*, *Aspergillus kawakii* and *Aspergillus tubigensis* (Figure 1), with temperature optimum of these xylanases around 45-55°C (Sunna and Antranikian, 1997). Therefore, the present invention also pertains to a modified xylanase comprising a non-aromatic hydrophobic amino acid, for example but not limited to Cys at position 118, and to a native xylanase comprising a non-aromatic hydrophobic amino acid at position 118, providing that the native xylanase exhibits a MET between about 69°C to about 84°C.

Another mutation at position 11 to Asp also benefits thermophilicity of xylanase. Mutant TrX-H-**11D**-ML-75A105H-125A129E-144R161R (Trx-H11D-ML-AHAE-RR) exhibits greater activity at higher temperatures, as compared to the precursor TrX-HML-75A105H-125A129E-144 (TrX-HML-AHAE; Figure 8). This result is also unexpected since (Turenen et. al. (2001) reported that the same N11D mutation lowered the temperature optima and range in a TrX mutant containing an intramolecular disulfide bond. Furthermore, US 5,759,840 discloses that the 11D mutation has no effect on thermophilicity of TrX-H-11D-ML (mutant termed NI-TX12). Therefore, the present invention also pertains to a native or a modified xylanase comprising an acidic amino acid, for example but not limited to Asp, at position 118, providing that the native or modified xylanase exhibits a MET between about 69°C to about 84°C.

Furthermore, mutations identified above can be combined to create mutant xylanases with greater thermophilicity, even at higher pH range. The combination mutants xylanases based on triple mutations N11D/ D116G/144R or N11D/Y118C/144R, namely:
TrX-H-**11D**-ML-75A105H-**116G**-125A129E-**144R**161R (TrX-H11D-ML-AHGAE-RR); and

TrX-H-11D-ML-75A105H-118C-125A129E-144R161R (Trx-H11D-ML-AHCAE RR), exhibited a maximum enzymatic activity at higher temperature of 70-75°C and further showed significant enzymatic activity at 80°C at pH 5.5 (Figure 5, only 116G mutant) and pH 6.0 (Figures 6 and 7). These results suggest the effects of the mutations D116G or Y118C with N11D and H144R on the thermophilicity of the mutant xylanase are complementary. Therefore, the present invention relates to a native or a modified xylanase comprising an acidic amino acid at position 11, a non-polar amino acid at position 116, and a basic amino acid at position 144, for example but not limited to N11D/ D116G/144R, or an acidic amino acid at position 11, a non-aromatic hydrophobic amino acid at position 118, and a basic amino acid at position at position 114, for example but not limited to N11D/Y118C/144R. Preferably the native or modified xylanase comprising an acidic amino acid at position 11, a non-polar amino acid at position 116, and a basic amino acid at position 144, or the xylanase comprising an acidic amino acid at position 11, a non-aromatic hydrophobic amino acid at position 118, and a basic amino acid at position at position 114, exhibits a MET between about 69°C to about 84°C.

In addition to achieve optimal activity at higher temperatures, the mutant xylanases based on the present invention, for example:
TrX-H-11D-ML-75A105H-116G-125A129E-144R161R (TrX-H11D-ML-AHGAE-RR); and
TrX-H-11D-ML-75A105H-118C-125A129E-144R161R (TrX-H11D-ML-AHCAE-RR),
(Figures 5 and 6), also demonstrate higher enzymatic activity (detected as greater xylose release) at their temperature optima. Both TrX-H11D-ML-AHGAE-RR and TrX-H11D-ML-AHCAE-RR exhibit about 600% activity at their temperature optima, than the activity observed at 40°C. This compared with the precursor, modified xylanase TrX-HML-75A105H-125A129E, which exhibits about 400% activity at its temperature optima, versus its activity at 40°C, and natural TrX (150% of its activity at its optimal temperature, v. value at 40°C).

This invention therefore includes a modified xylanase comprising a His at positions 10 and 105, a Met at position 27, a Leu at position 29, an Ala at positions 75 and 125, a Glu at position 129, and at least one of:

- an acidic amino acid at positions 11;
- a small non-polar amino acid at position 116;
- a medium-size non-aromatic hydrophobic amino acid at position 118; and
- a basic amino acid at position 144

Preferably, the amino acid at postion 11 is Asp (D), the amino acid at position 116 is Gly (G), the amino acid at position118 is Cys (C), and the amino acid at position 144 is selected from the group consisting of Lys (L), and Arg (R).

Increasing the Alkalophilicity of Xylanase

The effect of pH conditions on the enzymatic activity by the mutation Q161R in the mutant xylanase TrX-HML-75A105H-125A129E-144R**161R** (TrX-HML-AHAE-RR), is shown in Figure 9. Compared to its precursors TrX-HML-75A105H-125A129E and TrX-HML-75A105H-125A129E-144R (not shown). These latter enzymes have identical pH/activity profiles, however, the mutant xylanase TrX-HML-75A105H-125A129E-144R**161R** (TrX-HML-AHAE-RR) exhibits a greater activity at higher pH ranges of about 6.5 to about 8.0. TrX-HML-AHAE-RR also exhibits lower activity at lower pHs of about 5.0 to about 6.0, when compared to precursors without this mutation. Therefore, the present invention relates to a native or a modified xylanase comprising a basic amino acid, for example but not limited to Arg, at position 161. Preferably the native or modified xylanase with the basic amino acid at position 161 exhibits a MEP between about 5.8 to about 8.4.

The mutations at positions 116 and 118 to Gly and Cys, respectively, also improve enzymatic activity at higher pH ranges. Compared to native TrX, the single mutants TrX-**116G** and TrX-**118C** have greater activity at higher pH as shown in Figure 10.

The improvement by the mutation at positions 116 to a small non-polar residue to improve alkalophilicity is unexpected as no natural possess a Gly at position 116. Therefore, the present invention provides a native or a modified xylanase comprising a non-polar amino acid, for example but not limited to Gly, at position 116. Preferably the native or modified xylanase with the non-polar amino acid at position 116 exhibits a MEP between about 5.8 to about 8.4.

The improvement of thermophilicity based on the mutation at position 118 to cysteine is also unexpected, as most xylanases including the alkalophilic xylanase (for example, Tf, Bp, see Figure 1) possess a tyrosine (Y, 60%, Figure 1) and trytophan (W, 10%, Figure 1). The only xylanases possessing cysteine at position 118 are among the acidophilic *Aspergillus niger*, *Aspergillus kawakii* and *Aspergillus tubigensis* (Figure 1), with pH optimum of these xylanases around 2-4 (Sunna and Antranikian, 1997; Kinoshita et al 1995). Therefore, the present invention embraces a native or a modified xylanase comprising a non-aromatic hydrophobic

amino acid, for example but not limited to Cys, at position 118. Preferably the native or modified xylanase with the non-aromatic hydrophobic amino acid at position 118 exhibits a MEP between about 5.8 to about 8.4.

An enhancing effect in alkalophilicity of xylanase, by the mutations D116G and Y118C, is also observed in the mutants:
TrX-HML-75A105H-**116G**-125A129E-144R (TrX-HML-AHGAE-R); and
TrX-HML-75A105H-**118C**-125A129E-144R (TrX-HML-AHCAE-R),
(Figure 9), when compared to the precursor xylanases TrX-HML-75A105H-125A129E-144R and TrX-HML-75A105H-125A129E. While both mutants demonstrated higher activity at pH from about 6.5 to about 8.0, only the mutant TrX-HML-75A105H-**116G**-125A129E-144R (TrX-HML-AHGAE-R) retains substantially optimal activity at the lower pH of about 5.0 to about 6.0. This maintenance of high activity at pH of about 5.0 to about 8.0 represents a broadening of the optimal pH range by this mutation at position 116.

Mutations identified above have been combined to create mutant xylanases with greater alkalophilicity and thermophilicity. The combination mutants xylanases based on quadriple mutations N11D/D116G/H144R/Q161R or N11D/Y118C/144R/Q161R, namely:
TrX-H-**11D**-ML-75A105H-**116G**-125A129E-**144R161R** (TrX-H11D-ML-AHGAE-RR; Figure 9); and
TrX-H-**11D**-ML-75A105H-**118C**-125A129E-**144R161R** (TrX-H11D-ML-AHCAE-RR; not shown),
exhibit close to maximum enzymatic activity at pH from about 5.0 to about 7.0, as compared to their precursors. Furthermore the presence of the mutation D116G helps the retaining of substantially maximal activity at lower pH range of about 5.0 to about 6.0, thus avoiding the significant loss of activity at low pH observed in precursor TrX-HML-75A105H-125A129E-**144R161R** (Figure 9). This result further confirmed the broadening of the optimal pH range by this mutation at position 116. Therefore, the present invention relates to a native or a modified xylanase comprising an acidic amino acid at position 11, a non-polar amino acid at position 116, and a basic amino acid at position 114, for example but not limited to N11D/ D116G/144R, or an acidic amino acid at position 11, a non-aromatic hydrophobic amino acid at position 118, and a basic amino acid at position at position 114, for example but not limited to N11D/Y118C/144R. Preferably the native or modified xylanase comprising an acidic amino acid at position 11, a non-polar amino acid at position 116, and a basic amino acid at position 114, or the xylanase comprising an acidic amino acid at position 11, a non-aromatic hydrophobic amino acid at

position 118, and a basic amino acid at position at position 114 a MEP between about 5.8 to about 8.4.

This invention also provides a modified xylanase comprising a His at positions 10 and 105, a Met at position 27, a Leu at position 29, an Ala at positions 75 and 125, a Glu at position 129, and at least one of:

- an acidic amino acid at position 11;
- a small non-polar amino acid at position 116;
- a medium-size non-aromatic hydrophobic amino acid at position 118;
- a basic amino acid at position 161.

Preferably, the amino acid at postion 11 is Asp, the amino acid at position 116 is Gly, the amino acid at position118 is Cys, the amino acid at position 161 is selected from the group consisting of Lys, and Arg.

In summary, improved alkalophilic mutant TrX xylanases may be constructed through:

i) mutation of Asp 116 to a small non-polar residue, for example, but not limited to Gly;
ii) mutation of Tyr 118 to a medium-size, non-aromatic hydrophobic residue such as but not limited to Cys;
iii) mutation of Glen 161 to a basic amino acid Arg or Lys;
iv) combination of mutations described in i) with those described in ii) to iii) for the improvement of thermophilicity and alkalophilicity; or
v) combination of mutations described in i) to iv), above, with the HML series of mutations as described above (see U.S. 5,759,840 which is incorporated herein by refrence for HML mutations).

The above description is not intended to limit the claimed invention in any manner, furthermore, the discussed combination of features might not be absolutely necessary for the inventive solution.

Examples

The present invention will be further illustrated in the following examples. However, it is to be understood that these examples are for illustrative purposes only, and should not be used to limit the scope of the present invention in any manner.

EXAMPLE 1: Construction of *Trichoderma reesei* mutant xylanases

Basic recombinant DNA methods like plasmid preparation, restriction enzyme digestion, polymerase chain reaction, oligonucleotide phosphorylation, ligation, transformation and DNA hybridization were performed according to well-established protocols familiar to those skilled in the art (e.g. Sung et al., 1986) or as recommended by the manufacturer of the enzymes or kit. The buffers for many enzymes have been supplied as part of a kit or made according to the manufacturer's instructions. Restriction enzymes, T4 polynucleotide kinase and T4 DNA ligase were purchased from New England BioLabs Ltd, Mississauga, Ont. GeneAmp PCR reagent kit was purchased from Perkin-Elmer. A precursor plasmid pXYbc, which is a pUC type plasmid with a *Bacillus circulans* xylanase gene inserted, has previously been prepared and published (Sung et al, 1993; Campbell et al., U.S. Pat. No. 5,405,769). A commonly used *E. coli* strain, HB101 (Clonetech Lab, Palo Alto, CA) was used as a transformation and expression host for all gene constructs. Birchwood xylan and Remazol Brilliant Blue R-D-Xylan were purchased from Sigma (St. Louis, Mo). Hydroxybenzoic acid hydrazide (HBAH) was purchased from Aldrich. Oligonucleotides were prepared with an APPLIED BIOSYSTEM DNA synthesizer (model 380B). All xylanase enzymatic assays were performed in a covered circulating water bath (Haake type F 4391) and maintained within a temperature range of $\pm$ 0.1° C.

1-1: Construction of precursor plasmid pTrX harbouring synthetic TrX (SEQ ID NO: 39)

The precursor plasmid pTrX for mutations disclosed below has been previously published (Sung et al, 1995). This plasmid is derived from a pUC119 plasmid with a synthetic nucleotide sequence encoding a *Trichoderma reesei* xylanase (TrX; Figure 2). Expression of this xylanase and other mutant xylanases subsequently described are under the control of the *lac Z* promoter of the pUC plasmid. The total assembly of the *Trichoderma* xylanase gene required two stages, initially for the (92-190; Tr2 numbering) region, then followed by the (1-92; Tr2 numbering) region. The protocol for the construction of this gene is routine and identical to the standard published procedure for many other genes. The protocol requires enzymatic phosphorylation of overlapping synthetic oligonucleotides which encodes a xylanase. This is followed by their ligation into an appropriately cut plasmid.

For the construction of TrX (92-190), ten overlapping oligonucleotides (see Figure 2):

XyTv-101, SEQ ID NO:29;
XyTv-102, SEQ ID NO:30;
TrX-103, SEQ ID NO:31;
XyTv-104, SEQ ID NO:32;
XyTv-105, SEQ ID NO:33;
XyTv-106, SEQ ID NO:38;
XyTv-107, SEQ ID NO:37;
TrX-108, SEQ ID NO:36;
XyTv-109, SEQ ID NO:35; and
XyTv-110, SEQ ID NO:34

were designed with codon usage frequency imitating that of *E. coli*. The SalI and BglII cohesive ends of two terminal oligonucleotides enabled the enzymatic ligation of the ten fragments into the linearized plasmid pXYbc. The ten oligonucleotides (50 pmol, 1 μL for each) encoding the TrX(92-190) region of *Trichoderma* xylanase were phosphorylated in a mixture containing 10X standard kinase buffer (0.4 μL), 1 mM ATP (4 μL), T4 DNA kinase (5 units), and water (3 μL). Phosphorylation reactions were carried out for 1 h at 37° C. The solutions were then combined and heated to 70° C for 10 min. After being cooled slowly to room temperature, the combined solutions were added to a mixture of 4 mM ATP (3.5 μL), EcoR1-HindIII linearized plasmid pUC119 (0.1 pmol), and T4 DNA ligase (3.5 μL) and incubated at 12° C for 20 h. Aliquots of the ligation mixture were used to transform *E. coli* HB101 on YT plates (8 g yeast extract, 5 g bacto-tryptone, 5 g NaCl, 15 g of agar in 1 L of water) containing ampicillin (100 mg/L).

For the preparation of a hybridization probe, one of the oligonucleotides, for example XyTv-110 (10 pmol, 1 μL) was phosphorylated with ^{32}P-ATP (10 pmol, 3 μL) using T4 DNA kinase (1 μL), 10X kinase buffer (1 μL), and water (4 μL) at 37° C for 1 h.

Transformants were selected randomly for hybridization analysis. Colonies were grown on YT plates with ampicillin overnight, and transferred onto nylon filters. They were then denatured with 0.5N NaOH - 1.5M NaCl (10 min) and neutralized with 0.5N Tris-HCl (pH 7.0) - 1.5M NaCl (10 min). After ultraviolet irradiation at 254 nm for 8 min, the filters were washed with 6X SSC - 0.05% Triton X-100 for 30 min. Cell debris was scraped off completely. After another 30 min. in fresh solution, duplicate filters were transferred individually into separate mixtures of 6X SSC - 1% dextran sulphate - 0.05% TritonX-100 - 1X Denhardt's hybridization

fluid. The ^{32}P-labelled probe was added to the filter. After 16 h at 45° C, the filter was washed twice with 6X SSC - 0.05% TritonX-100 at room temperature for 5 min. and then at 65° C for 30 min. Positively hybridized clones with the intermediate plasmid pBcX-TrX were identified by auto-radiographic analysis.

The above protocol, involving enzymatic phosphorylation of synthetic overlapping oligonucleotides and ligation into a linearized plasmid, was employed in the assembly of the TrX(1-92) region and in the cassette mutagenesis for the subsequent generation of other mutant xylanases described in this invention.

For the assembly of the TrX(1-92; Tr2 numbering) region to complete the full-length *Trichoderma reesei* xylanse II gene (TrX), the intermediate plasmid pBcX-TrX was linearized by NheI and KpnI endonucleases to release the DNA insert for BcX(1-83). With NheI and KpnI cohesive ends, eight overlapping oligonucleotides:

TrX-1,	SEQ ID NO: 21;
XyTv-2,	SEQ ID NO:22;
TrX-3,	SEQ ID NO:23;
XyTv-4,	SEQ ID NO:24;
XyTv-5,	SEQ ID NO:28;
TrX-6,	SEQ ID NO:27;
XyTv-7,	SEQ ID NO:26; and
TrX-8	SEQ ID NO:25,

encoding the TrX(1-91) sequence were ligated into the linearized plasmid pBcX-TrX (Figure 2), via the protocol described above. The new plasmid pTrX therefore harbored a synthetic TrX gene (SEQ ID NO:39).

All mutant xylanase genes described below have been constructed via the method of cassette mutagenesis. The protocol for cassette mutagenesis was identical to that described for gene assembly described above. Generally, cassette mutagenesis involved (i) enzymatic phosphorylation of overlapping synthetic oligonucleotides, (ii) ligation of synthetic oligonucleotides with a linearized plasmid, (iii) transformation of the plasmid into *E. coli* HB101 competent cells, (iv) identification of mutant transformants via hybridization with the labelled oligonucleotide, and (v) confirmation of the mutation through dideoxy nucleotide sequencing.

1-2: Construction of the precursor plasmid pTrX-HML

The construction of this precursor plasmid pTrX-HML has been described in detail in U.S. Pat. No. 5,759,840 (see Example 1N, herein incorporated by reference; plasmid termed pNI-TX13). TrX-HML comprises the native TrX xylanase, along with three mutations at N10H (Asn at position 10 is replaced with His), Y27M and N29L. The first thirty amino acids of the sequence comprising N10H, Y27M and N29L are shown below.

```
TrX                                    1   2   3   4   5   6   7   8
amino acid                             Q   T   I   Q   P   G   T   G
5'-CT AGC TAA GGA GG CTG CAG ATG CAA ACA ATA CAA CCA GGA ACC GGT
    3'-G ATT CCT CC GAC GTC TAC GTT TGT TAT GTT GGT CCT TGG
CCA
    NheI                                                    PinAI

  9  10  11  12  13  14  15  16  17  18  19  20  21  22  23  24
  Y   H   N   G   Y   F   Y   S   Y   W   N   D   G   H   G   G
TAC CAC AAC GGT TAC TTT TAC AGC TAT TGG AAC GAT GGC CAT GGA GGC
ATG GTG TTG CCA ATG AAA ATG TCG ATA ACC TTG CTA CCG GTA CCT CCG

 25  26  27  28  29  30
  V   T   M   T   L   G
GTC ACA ATG ACT CTG GGG
CAG TGT TAC TGA GAC CCC
```

1-3: Construction of the deletion plasmid pTrX-HML-(1-113)

Plasmid pTrX-HML-(1-113) comprises the amino acid sequence 1-113 of TrX (SEQ ID NO:39) and cannot express an active xylanase. Such transformants are confirmed by the absence of a clearing zone or halo around the transformant colonies on blue xylan plates.

The new plasmid was constructed via (i) the removal of the TrX(114-190) coding sequence of pTrX-HML through cutting with restriction enzymes BamHI and BglII, (ii) ligation of the identical cohesive ends of the linearized plasmid, (iii) transformation into the *E. coli* HB101 competent cells followed by platting on YT plate (containing 5 g yeast extract, 3 g bacto-

tryptone, 5 g NaCl, 15 g of agar in 1 L of water, 1 g Remazol Brilliant Blue R-D-xylan) and ampicillin (100 mg/L), (iv) identification of the mutant transformants through the loss of xylanase activity (absence of a clearing zone or halo around the colonies on the blue xylan plate overnight at 40°C), and (v) confirmation of the mutation through dideoxy nucleotide sequencing. The protocol for each of these steps was similar to that for gene assembly described above.

1-4: Construction of the plasmid pTrX-HML-105R

Mutant xylanase pTrX-HML-105R is similar to TrX-HML except that Leu at position 105 is replaced by Arg (L105R).

PCR was used to generate a DNA fragment encoding (100-190) region with the L105R mutation. The PCR primers with mutation (in bold type) in the construction of pTrX-HML-105R is shown below:

TX-105R-1 (SEQ ID NO:44)

```
100 101 102 103 104 105 106 107 108 109 110 111 112 113
  T   G   A   T   K   R   G   E   V   T   S   D   G   S
5'-ACC GGC GCC ACA AAA AGA GGC GAA GTC ACT AGT GAT GGA TCC
       KasI
```

Reverse PCR primer TX-C1 comprised:

TX-C1 (SEQ ID NO:42)

```
183 184 185 186 187 188 189 190  ter
  G   S   A   S   I   T   V   S
CCA AGG CGA TCA TAA TGT CAC TCG ATT TCT AGA ACT TCG AAC CC-5'
                                  BglI     HindIII
```

The appropriate PCR template and primers, and the restriction enzymes to cut the end of the PCR products are listed below (Table 3-1).

Table 3-1

PCR product	PCR upstream primer	PCR reverse Primer	PCR template	Restriction enzymes for PCR product
(a)	TX-105R-1	TX-C1	pTrX	KasI/ HindIII

The cut PCR product (a) (Table 3-1) was ligated into a KasI/HindIII-linearized plasmid pTrX-HML(1-113) to generate plasmid pTrX-HML-105R.

1-5: Construction of the plasmids pTrX-HML-75A105R and pTrX-HML-75G105R

Xylanase mutants TrX-HML-75A-105R and TrX-HML-75G-105R are similar to TrX-HML-105R, with the exception of an additional single mutation S75A or S75G respectively.

The PCR primers with mutations S75A (TX-75A-1; SEQ ID NO: 40) and S75G (TX75-G-1; SEQ ID NO: 46) are shown below.

TX-75A-1 (SEQ ID NO:40)

```
 69  70  71  72  73  74  75  76  77  78  79  80  81
  N   G   N   S   Y   L   A   V   Y   G   W   S   R
5'-T GGG AAT TCA TAC TTA GCC GTC TAT GGC TGG TCT AG
      EcoRI
```

TX-75G-1 (SEQ ID NO:46)

```
 69  70  71  72  73  74  75  76  77  78  79  80  81
  N   G   N   S   Y   L   G   V   Y   G   W   S   R
5'-T GGG AAT TCA TAC TTA GGC GTC TAT GGC TGG TCT AG
      EcoRI
```

The appropriate PCR template and primers, and the restriction enzymes to cut the end of the PCR products are listed below (Table 3-2).

Table 3-2

PCR product	PCR upstream primer	PCR reverse Primer	PCR template	Restriction enzymes for PCR product
(b)	TX-75A-1	TX-C1	pTrX-HML-105R	EcoRI/ HindIII
(c)	TX-75G-1	TX-C1	pTrX-HML-105R	EcoRI/ HindIII

The EcoRI/HindIII-cut PCR products (b) and (c) (see Table 3-2) were prepared and ligated into EcoRI/HindIII-linearized pTrX-HML(1-113) plasmid to generate plasmids pTrX-HML-75A-105R and pTrX-HML-75G-105R respectively.

1-6: Construction of the plasmid pTrX-HML-75G105R-125A129E

The mutant TrX-HML-75G-105R-125A129E was identical to TrX-HML-75G-105R, with the exception of the additional mutations Q125A and I129E.

The intact mutant xylanase gene was assembled via the ligation of two DNA sequences encoding the 1-121 and the 122-190 regions. The DNA sequecence encoding the 1-121 region was isolated through the deletion of plasmid pTrX-HML-75G-105R with restriction nucleases listed below (Table 3-3).

Table 3-3

Deletion sequence	Precursor plasmid	Restriction enzymes
(A)	pTrX-HML-75G-105R	NheI/ MluI

The DNA sequence encoding the 122-190 region was a PCR product (d) by a primer encoding the mutations as shown below.

TX-125A129E-1 (SEQ ID NO:49)

```
120 121 122 123 124 125 126 127 128 129 130 131 132 133
     Q   R   V   N   A   P   S   I   E   G   T   A   T
5'-C CAA CGC GTT AAT GCG CCA TCG ATC GAG GGA ACC GCC ACC
     MluI
```

The appropriate PCR template and primers, and the restriction enzymes to cut the end of the PCR product, are listed below (Table 3-4).

Table 3-4

PCR product	PCR upstream primer	PCR reverse primer	PCR template	Restriction enzymes for PCR product
(d)	TX-125A129E-1	TX-C1	pTrX	MluI/ HindIII

The cut PCR product (d) and the deletion sequence (A) were ligated to the NheI/HindIII-linearized plasmid pTrX-(1-113) to generate the plasmid pTrX-HML-75G-105R-125A129E.

1-7: Construction of the plasmid pTrX-HML-75A105H-125A129E

The intact mutant gene was assembled via the ligation of two DNA sequences encoding the 1-101 and the 102-190 regions.

For the preparation of the DNA sequence encoding the 1-101 region, restriction nucleases for the deletion of the appropriate plasmid are listed below (Table 3-5).

Table 3-5

Deletion sequence	Precursor plasmid	Restriction enzymes
(B)	pTrX-HML-75A-105R	NheI/ KasI

For the preparation of the DNA sequence encoding the 102-190 region, polymerase chain reaction was used with primer TX-105H-1.

TX-105H-1 (SEQ ID NO:41)

```
   100 101 102 103 104 105 106 107 108 109 110 111 112 113
     T   G   A   T   K   H   G   E   V   T   S   D   G   S
5'-ACC GGC GCC ACA AAA CAC GGC GAA GTC ACT AGT GAT GGA TCC
       KasI
```

The appropriate PCR primers with mutations at position-105 and the restriction enzymes to cut the end of the PCR product are listed below (Table 3-6).

Table 3-6: Plasmid pTrX-HML-75G-105R-125A129E as PCR template.

PCR product	PCR upstream primer	PCR reverse primer	Restriction enzymes for PCR product
(e)	TX-105H-1	TX-C1	KasI/ HindIII

The cut PCR product (e) and the deletion sequence (B) were ligated to the NheI/HindIII-linearized plasmid pTrX-(1-113) to generate the plasmid pTrX-HML-75A-105H-125A129E.

1-8: Construction of the deletion plasmid pTrX-del(43-53)

A plasmid pTrX-del(43-53) encoding an inactive xylanase with the (43-53) region deleted ,was constructed via restriction cutting of the plasmid pTrX at the BspEI site at residue

43 and the XmaI site at residue-53 and self-ligation of the identical ends. After transformation, the correct clones were identified through non-expressing of xylanase or absence of halo or clearing zone in blue xylan-containing YT plates.

1-9: Construction of the deletion plasmids pTrX-del(123-144) and pTrX-HML-75A105H-del(123-144)

Two plasmids containing partially deleted xylanase gene, were constructed via a PCR reaction with a new primer encoding the deletion of the (123-144) region.

PCR oligonucleotide primers:

TX-del(123-144)-1r (SEQ ID NO:43)

```
148 147 146 145     122 121 120 119 118 117 116 115
  G   S   S   R       R   Q   T   R   Y   I   D   Y
5'-C GGA GCT CCG AC GCG TTG GGT ACG GTA GAT ATC ATA
     SacI          MluI
```

TX-N1 (SEQ ID NO:45)

```
                                  1   2   3   4   5   6   7
                                Q   T   I   Q   P   G   T
5'-CT AGC TAA GGA GG CTG CAG ATG CAA ACA ATA CAA CCA GGA A
   NheI           PstI
```

Table 3-7: PCR template with TX-del(123-144)-1r and TX-N1 as primer

PCR product	PCR template	Restriction enzymes for PCR product
(f)	pTrX	PstI/ SacI
(g)	PTrX-HML-75A105H-125A129E	PstI/ SacI

Ligation of the cut PCR fragments (f) and (g) to the PstI/SacI-linearized plasmid pTrX and transformation to yield the correct clones harboring the deletion plasmids pTrX-del(123-144) and pTrX-HML-75A105H-del(123-144) respectively, that were identified through non-

expressing of xylanase and absence of halo or clearing zone in the blue xylan-containing YT plates.

1-10: Construction of the plasmid pTrX-HML-75A105H-125A129E-144R

The new mutant pTrX-HML-75A105H-125A129E-144R differs from the precursor pTrX-HML-75A105H-125A129E by an additional mutation H144R. A new PCR reverse primer was used to create this mutation.

TX-144R-1r (SEQ NO:47)

```
   159 158 157 156 155 154 153 152 151 150 149 148 147 146 145
144 143 142
     W   A   N   F   H   N   A   T   N   V   S   G   S   S   R
R   N   R 5'-CCA TGC ATT AAA GTG ATT CGC AGT ATT AAC CGA ACC GGA
GCT CCG ACG ATT ACG
     NsiI

141 140 139 138
  R   V   S   W
TCT AAC ACT CCA
```

The appropriate PCR template and primers, and the restriction enzymes to cut the end of the PCR product which is the 1-146 sequence, are listed below (**Table 3-8**).

Table 3-8

PCR product	Upstream primer	Downstream primer	Template	Restriction cut
(h)	TX-N1	TX-144R-1r	pTrX-HML-75A105H-125A129E	PstI/ NsiI

The PstI/NsiI-cut PCR fragment (h) was ligated to the PstI/NsiI-linearized plasmid pTrX-del(43-53) to restore the functional xylanase gene in the new plasmid pTrX-HML-75A-105H-125A-129E-144R.

1-11: Construction of the plasmid pTrX-HML-75A105H-125A129E-144R161R

The new mutant pTrX-HML-75A105H-125A129E-144R161 differs from the precursor pTrX-HML-75A105H-125A129E-144R by an additional mutation Q161R. A new PCR reverse primer was used to create this mutation.

TX-161R-1r (SEQ ID NO: 48)

```
168 167 166 165 164 163 162 161 160 159 158 157 156 155 154
  T   G   L   T   L   G   Q   R   A   W   A   N   F   H   N
5'-GT ACC TAG GGT TAA CCC TTG CCG TGC CCA TGC ATT AAA GTG ATT
      AvrII
```

A PCR product encoding the TrX(1-165) region was prepared as described in Table 3-9.

Table 3-9: Plasmid pTrX-HML-75A-105H-125A129E-144R as PCR template.

PCR product	PCR primer	upstream PCR reverse primer	Restriction enzymes for PCR product
(i)	TX-N1	TX-161R-1r	PstI/ AvrII

The PstI/AvrII-cut PCR fragment (i) was ligated to the PstI/AvrII-linearized plasmid pTrX-del(43-53) to restore the functional xylanase gene in the new plasmid pTrX-HML-75A-105H-125A-129E-144R161R.

1-12: Construction of the plasmids pTrX-116G and pTrX-118C

The two new mutants are identical to TrX, with the major difference of an additional mutation, i.e. Asp-116 to Gly (D116G) or Tyr-118 to Cys (Y118C).

Two PCR primers were prepared with mutation (in bold type).

```
                TX-116G-1 (SEQ ID NO:50)
   111 112 113 114 115 116 117 118 119
     D   G   S   V   Y   G   I   Y   R
5'-GAC GGA TCC GTA TAT GGT ATC TAC CG
       BamHI

                TX-118C-1 (SEQ ID NO:51)
   111 112 113 114 115 116 117 118 119 120 121 122
     D   G   S   V   Y   D   I   C   R   T   Q   R
5'-GAC GGA TCC GTA TAT GAT ATC TGC CGT ACC CAA CGC
       BamHI
```

The following plasmid template and primers are required for the two PCR:

Table 3-10: PCR with plasmid pTrX as template

PCR product	PCR upstream primer	PCR reverse primer	Restriction cuts
(j)	TX-116G-1	TX-C1	BamHI/HindIII
(k)	TX-118C-1	TX-C1	BamHI/HindIII

Ligation of the cut PCR products (j) and (k) to BamHI/HindIII-linearized plasmid pTrX-del(123-144) restored a functional xylanase gene in transformants harboring the respective plasmids pTrX-116G and pTrX-118C.

1-13: Construction of the plasmids pTrX-HML-75A105H-116G-125A129E-144R and pTrX-HML-75A105H-118C-125A129E-144R

The two new mutants were identical to the precursor TrX-HML-75A105H-125A129E-144R, with the major difference of an additional mutation, i.e. Asp-116 to Gly (D116G) or Tyr-118 to Cys (Y118C).

The following plasmid template and primers are required for the two PCR:

Table 3-11: PCR with plasmid pTrX-HML-75A105H-125A129E-144R as template

PCR product	PCR upstream primer	PCR reverse primer	Restriction cuts
(l)	TX-116G-1	TX-C1	BamHI/HindIII
(m)	TX-118C-1	TX-C1	BamHI/HindIII

Ligation of the cut PCR products (l) and (m) to the BamHI/HindIII-linearized plasmid pTrX-HML-75A105H-del(123-144) restored a functional xylanase gene in transformants harboring the respective plasmids pTrX-HML-75A105H-116G-125A129E-144R and pTrX-HML-75A105H-118C-125A129E-144R.

1-14: Construction of the plasmids pTrX-H-11D-ML-75A105H-125A129E-144R161R, pTrX-H-11D-ML-75A105H-116G-125A129E-144R161R and pTrX-H-11D-ML-75A105H-118C-125A129E-144R161R

The new mutants TrX-H-11D-ML-75A105H-125A129E-144R161R, TrX-H-11D-ML-75A105H-116G-125A129E-144R161R and TrX-H-11D-ML-75A105H-118C-125A129E-144R161R were identical to their respective precursors TrX-HML-75A105H-125A129E-144R, TrX-HML-75A105H-116G-125A129E-144R and TrX-HML-75A105H-118C-125A129E-144R, with the major difference of additional mutations, i.e. Asn-11 to Asp (N11D) and Gln-161 to Arg (Q161R). A new PCR primers was prepared with mutation N11D (in bold type).

TX-10H11D-1 (SEQ NO:52)

```
     6   7   8   9  10  11  12  13  14  15  16  17  18
     G   T   G   Y   H   D   G   Y   F   Y   S   Y   W
5'-GGA ACC GGT TAC CAC GAC GGT TAC TTT TAC AGC TAT TGG
       AgeI
```

Table 3-13

PCR product	Upstream primer	Downstream primer	Template	Restriction cut
(n)	TX-10H11D-1	TX-161R-1r	pTrX-HML-75A105H-125A129E-144R	AgeI/ AvrII
(o)	TX-10H11D-1	TX-161R-1r	pTrX-HML-75A105H-116G-	AgeI/ AvrII

			125A129E-144R	
(p)	TX-10H11D-1	TX-161R-1r	pTrX-HML-75A105H-118C-125A129E-144R	AgeI/ AvrII

Ligation of the cut PCR products (n), (o) and (p) to AgeI/AvrII-cut plasmid pTrX-del(43-53) restored a functional xylanase gene in the transformant harboring the new plasmids pTrX-H-11D-ML-75A105H-125A129E-144R161R, pTrX-H-11D-ML-75A105H-116G-125A129E-144R161R and pTrX-H-11D-ML-75A105H-118C-125A129E-144R161R respectively

1-15: Construction of the deletion plasmid pTrX-H-11D-ML-75A105H-116G-del(123-144)

A plasmid containing partially deleted xylanase gene, were constructed via a PCR reaction with a new primer encoding the deletion of the (123-144) region, via a protocol identical to the **EXAMPLE 1-9.**

Table 3-14: PCR template with TX-del(123-144)-1r and TX-N1 as primer

PCR product	PCR template	Restriction enzymes for PCR product
(q)	pTrX-H-11D-ML-75A105H-116G-125A129E-144R161R	PstI/ SacI

Ligation of the cut PCR fragment (q) to the PstI/SacI-linearized plasmid pTrX and transformation to yield the correct clones harboring the deletion plasmid pTrX-H-11D-ML-75A105H-116G-del(123-144), that were identified through non-expressing of xylanase and absence of halo or clearing zone in the blue xylan-containing YT plates.

1-16: Construction of the plasmid pTrX-H-11D-ML-75A105H-116G118C-125A129E-144R161R

The new mutant TrX-H-11D-ML-75A105H-116G118C-125A129E-144R161R was identical to its precursors TrX-H-11D-ML-75A105H-116G-125A129E-144R161R and TrX-H-11D-ML-75A105H-118C-125A129E-144R161R with the difference in the possession of combination mutation, Tyr-118 to Cys (Y118C) and Asp-116 to Gly (D116G). A new PCR primers was prepared with the combination mutation D116G / Y118C (in bold type).

TX-116G118C-1 (SEQ ID NO:53)

```
111 112 113 114 115 116 117 118 119 120 121 122
  D   G   S   V   Y   G   I   C   R   T   Q   R
5'-GAC GGA TCC GTA TAT GGT ATC TGC CGT ACC CAA CGC
      BamHI
```

Table 3-15. PCR to create (112-167) fragment containing the combination mutation

PCR product	Upstream primer	Downstream primer	Template	Restriction cut
(r)	TX-116G118C-1	TX-161R-1r	pTrX-HML-75A105H-125A129E-144R	BamHI/ AvrII

Ligation of the cut PCR products (r) to BamHI/AvrII-cut plasmid pTrX-H-11D-ML-75A105H-116G-del(123-144) restored a functional xylanase gene in the transformant harboring the new plasmid pTrX-H-11D-ML-75A105H-116G118C-125A129E-144R161R.

Example 2: Characterization of mutant xylanases

2-1: Production of xylanases

The culture conditions comprised a 5 ml culture of overnight innoculant in 2YT medium (16 g bacto-tryptone, 10 g yeast extract, 5 g NaCl, 1 L of water) containing ampicillin (100 mg/L) was added to 2YT medium (1 L) with ampicillin. The cultures were grown with shaking (200 rpm) at 37°C. After 16 hr, cells were harvested.

2-2: Purification of mutant xylanases

Protein samples were prepared from cells by first making an extract of the cells by grinding 10 g of the cell paste with 25 g of alumina powder. After grinding to smooth mixture, small amounts (5 mL) of ice cold buffer A (10mM sodium acetate, pH 5.5 for BcX mutants) or buffer B (10mM sodium acetate, pH 4.6 for TX mutants) were added and the mixture ground vigorously between additions. The alumina and cell debris were removed by centrifugation of the mixture at 8000 x g for 30 min.

Prior to column chromatography, the supernatant was adjusted to pH 4.6 by acetic acid and centrifuged to remove any precipitate. The subsequent method for column chromatography was identical for all mutant xylanases.

Following acidification and centrifugation, the xylanase sample was pumped onto a 50 ml bed volume, CM-sepharose fast flow, cation exchange column (Pharmacia Biotech, Uppsala), equilibrated in 10 mM sodium acetate (pH 4.6). The xylanase was eluted with a 250 ml linear gradient (0 to 0.6M NaCl in 10 mM sodium acetate , pH 4.6) at a flow rate of 1 ml/min. The xylanases elute at 150 to 200 ml of the gradient. Aliquots from the collected fractions are examined by SDS-PAGE, and those fractions having most of the xylanase present were pooled. The purified xylanase was quantified by spectrophotometry at 280 nm using an extinction coefficient between 54,600 - 53,400 M^{-1} for most mutant TrX xylanases. A typical purification from 10g of cells yielded 25 mg of xylanase.

2-3: Standard assay for the measurement of enzymatic activity

The quantitative assay determined the number of reducing sugar ends generated from soluble xylan. The substrate for this assay was the fraction of birchwood xylan which dissolved in water from a 5% suspension of birchwood xylan (Sigma Chemical Co.). After removing the insoluble fraction, the supernatant was freeze dried and stored in a dessicator. The measurement of specific activity was performed as follows: Reaction mixtures containing 100 µL of 30 mg/mL xylan previously diluted in assay buffer (50 mM sodium citrate, pH 5.5 or the pH optimum of the tested xylanase), 150 µL assay buffer, and 50 µL of enzyme diluted in assay buffer were incubated at 40° C. At various time intervals 50 µL portions were removed and the reaction stopped by diluting in 1 mL of 5 mM NaOH. The amount of reducing sugars was determined with the hydroxybenzoic acid hydrazide reagent (HBAH) (Lever, 1972, Analytical Biochem 47:273-279). A unit of enzyme activity was defined as that amount generating 1 µ mol reducing sugar in 1 minute at 40° C.

For comparison of the specific activities between mutant and native xylanases the specific activities of a mutant xylanase was converted to a relative activity. The relative activity is calculated as a percentage, by dividing the specific activity of the mutant enzyme by the specific activity of the native xylanase.

Table 4: Relative activity of mutant and native xylanases at 40°C and pH 5.5.

Xylanase	Relative activity
native TrX	100*
TrX-HML-75A105H-**116G**-125A129E-144R	84
TrX-H-11D-ML-75A105H-**116G**-125A129E-144R161R	80
TrX-HML-75A105H-**118C**-125A129E-144R	113
TrX-H-11D-ML-75A105H-**118C**-125A129E-144R161R	121

* Specific activity of native TrX xylanase determined to be 770 U/mg.

The results depicted in Table 4 indicate that the specific enzymatic activities of the mutant xylanases at 40° C have not changed significantly as compared to the native xylanase. Rather, with the 118C mutant xylanases (TrX-HML-AHCAE-R, and TrX-H11D-ML-AHCAE-R) more activity is observed, when compared with the native xylanase (an increase in 13-21% in specific activity).

2-4: Determination of the expression efficiency of mutant xylanases by *E. coli*

Via the standard assay described in **2-3**, the relative expression efficiency for each mutant xylanase has been determined, via an estimation of xylose release by the xylanase produced in unit volume of the bacterial culture. The three mutant xylanases encoding the mutation N11D, namely:
TrX-H-**11D**-ML-75A105H-125A129E-144R161R (TrX-H11D-ML-AHAE-RR);
TrX-H-**11D**-ML-75A105H-116G-125A129E-144R161R (TrX-H11D-ML-AHGAE-RR); and
TrX-H-**11D**-ML-75A105H-118C-125A129E-144R161R (TrX-H11D-ML-AHCAERR)
are 2.4 - 4.3 fold more efficiently expressed by *E. coli*, as compared to their respective precursors TrX-HML-75A105H-125A129E-144R161R, TrX-HML-75A105H-116G-125A129E-144R and TrX-HML-75A105H-118C-125A129E-144R without this mutation.

Table 5: Expression efficiency of mutant xylanases

Xylanase	Relative expression efficiency*
TrX-H-**11D**-ML-75A105H-125A129E-144R161R	2.4 fold
TrX-H-**11D**-ML-75A105H-116G-125A129E-144R161R	3.1 fold
TrX-H-**11D**-ML-75A105H-118C-125A129E-144R161R	4.3 fold

* Relative to the respective precursors as stated in the text above.

This indicates that one of the benefits of the mutation N11D is the enhancement of expression in microbes, a important characteristic for the industrial production of the enzyme.

Example 3: Thermophilicity of mutant xylanases

Thermophilicity was examined to test the effect of different temperatures on the enzymatic hydrolysis of soluble xylan by different mutant xylanases.

The assay procedure was similar to the standard assay with changes in the incubation temperature and time. The xylanases (15 μg/mL) and soluble birchwood xylan substrate, in 50 mM sodium citrate buffer of pH 5.5, were mixed and incubated in a circulating water bath at different temperatures. After a 30-min incubation, the amount of reducing sugars released from xylan was determined by HBAH analysis and was calculated as a relative activity, with the value at 40°C representing 100%.

The effect of temperature on the hydrolysis of xylan by TrX-HML-75A105H-125A129E-**144R** (TrX-HML-AHAE-R) is shown in Figure 3. Compared to the presursor without the H144R mutation (TrX-HML-AHAE), this mutant xylanase showed a moderately improved enzymatic activity at higher temperature. These results suggest that the H144R mutation improves the thermophilicity of xylanases.

Another mutant TrX-HML-75A105H-125A129E-144R**161R** (TrX-HML-AHAE-RR) did not significantly enhance the enzymatic activity at higher temperature (not shown), as compared to TrX-HML-75A105H-125A129E-144R (TrX-HML-AHAE-R). These results suggest that the Q161R mutation does not benfit the thermophilicity of xylanases.

Two series of the mutants based on the mutations D116G and Y118C have been tested. Compared to native TrX, the single mutants TrX-**116G** and TrX-**118C** exhibit greater activity at higher temperatures (Figure 4).

The same enhancing effect in thermophilicity was also observed in the next pairs of mutants:
TrX-HML-75A105H-**116G**-125A129E-144R (TrX-HML-AHGAE-R); and
TrX-HML-75A105H-**118C**-125A129E-144R (TrX-HML-AHCAE-R),
as compared to the precursor TrX-HML-75A105H-125A129E-144R (Trx-HML-AHAE-R) at pH 5.5 (Figure 5, only 116G mutant shown) and pH 6.0 (Figures 6 and 7; these figures comprise the same data but have different representation of the relative activity).

Another series of mutant xylanases based on the N11D mutation also benefits the thermophilicty. Mutant TrX-H-**11D**-ML-75A105H-125A129E-144R161R (TrX-H11D-ML-AHAE-RR) exhibited greater activity at higher temperatures, as compared to the precursor TrX-HML-75A105H-125A129E-144R (TrX-HML-AHAE-R;Figure 8). This result is unexpected as prior art resports indicated the same N11D mutation either lowered the temperature optima and temperature range in TrX mutants containing an intramolecular disulfide bond (Turenen et. al., 2001), or no effect on thermophilicity of TrX-H-11D-ML was observed (US 5,759,840; mutant termed NI-TX12). These data of the present invention indicate that the 11D mutation benefits appropriately modified xylanases.

The mutations identified above can be combined to create mutant xylanases with greater thermophilicity, even at higher pH range. The combination mutant xylanases, comprising triple mutations N11D/ D116G/144R or N11D/Y118C/144R, namely:
TrX-H-**11D**-ML-75A105H-**116G**-125A129E-**144R**161R (TrX-H11D-ML-AHGAE-RR); and
TrX-H-**11D**-ML-75A105H-**118C**-125A129E-**144R**161R (TrX-H11D-ML-AHCAE-RR),
exhibit maximum enzymatic activity at a higher temperature of about 70 to about 75° C and show significant enzymatic activity at 80°C at pH 5.5 (Figure 5, only 116G mutant shown), and pH 6.0 (Figures 6 and 7). These results suggest the effects of the mutations, D116G or Y118C, complement the mutations, N11D and H144R, with respect to thermophilicity of xylanase.

Example 4: Alkalophilicity of mutant xylanases

The alkalophilicity of genetically modified xylanases was examined to test the effect that different pH conditions had on the enzymatic hydrolysis of soluble birchwood xylan by mutant

xylanases. The assay procedure was similar to the standard assay with changes in the incubation temperature and time. Aliquots of genetically modified xylanases (15 μg/mL) and soluble xylan substrate in 50 mM sodium citrate buffers which varied between pH 4-7 were incubated together at 65° C. Following 30 min incubations, the amount of reducing sugars released from the xylan substrate was determined by HBAH analysis and the enzymatic activity as a function of pH was calculated for a variety of mutant xylanases with the maximal activity taken as 100%.

The mutation H144R does not affect the activity at higher pH. The mutant TrX-HML-75A105H-125A129E-144R and its precursor TrX-HML-75A105H-125A129E have the same pH/activity profile (not shown). However, as noted in Example 3, this mutation (H144R) is beneficial to the thermophilicity of xylanase.

The effect of pH on the enzymatic activity by the mutation Q161R in the mutant xylanase TrX-HML-75A105H-125A129E-144R**161R** (TrX-HML-AHAE-RR) is shown in Figure 9. Compared to its precursors TrX-HML-75A105H-125A129E and TrX-HML-75A105H-125A129E-144R (not shown), both of which have identical pH/activity profiles, the mutant xylanase TrX-HML-75A105H-125A129E-144R**161R** (TrX-HML-AHAE-RR) exhibits greater activity at a higher pH range of 6.5, 7.0, 7.5 and 8.0. TrX-HML-AHAE-RR also exhibits lower activity at lower pH of 5.0, 5.5 and 6.0, when compared to precursors without this mutation.

The direct effect of the mutations D116G and Y118C on xylanase activity have been tested. Compared to native TrX, the single mutants TrX-**116G** and TrX-**118C** have demonstrated greater activity at higher pH (Figure 10).

The same enhancing effect in alkalophilicity by the mutations D116G and Y118C is also observed in the mutants:
TrX-HML-75A105H-**116G**-125A129E-144R (TrX-HML-AHGAE-R); and
TrX-HML-75A105H-**118C**-125A129E-144R (TrX-HML-AHCAE-R; Figure 9),
when compared to the precursors TrX-HML-75A105H-125A129E-144R and TrX-HML-75A105H-125A129E. While both of these mutants demonstrated higher activity at pH 6.5, 7.0, 7.5 and 8.0, the mutant TrX-HML-75A105H-**116G**-125A129E-144R retains optimal activity at the lower pH of 5.0, 5.5 and 6.0. This maintenance of high activity at pH 5.0- 8.0 by both of these mutants represents a broadening of the optimal pH range.

The N11D mutation does not appear to contribute to the alkalophilicity of TrX. The mutant TrX-H-**11D**-ML-75A105H-125A129E-144R161R (TrX-H11DML-AHAE-RR) has

identical pH/activity profile as its precursor TrX-HML-75A105H-125A129E-144R161R (not shown). The result, of 11D having no effect in the pH/activity profile, contradicts Turenen et al. (2001) who note that the N11D mutation lowered the pH optima and pH range in a TrX mutant containing an intramolecular disulfide bond. However, the results as described above agree with that in US 5,759,840, where no negative effect on alkalophilicity of TrX-H-11D-ML (mutant termed NI-TX12) was observed.

Mutations identified above have been combined to create mutant xylanases with greater alkalophilicity and thermophilicity. The combination mutants xylanases based on quadriple mutations N11D/D116G/H144R/Q161R or N11D/Y118C/144R/Q161R, namely:
TrX-H-11**D**-ML-75A105H-**116G**-125A129E-**144R161R** (TrX-H11D-ML-AHGAE-RR; Figure 9); and
TrX-H-11**D**-ML-75A105H-**118C**-125A129E-**144R161R** (TrX-H11D-ML-AHCAE-RR; not shown),
exhibit a maximum enzymatic activity from about pH 5.5 to about pH 7, as compared to precursor xylanases. Furthermore the presence of the mutation D116G helps the retain substantially maximal activity at a lower pH range of 5.0, 5.5 and 6.0, thus avoiding the significant loss of activity at low pH as observed in precursor TrX-HML-75A105H-125A129E-**144R161R** (Figure 9). This result further confirmes the broadening of the optimal pH range.

In summary, alkalophilic xylanase can be constructed through combination of mutations, D116G or Y118C with Q161R. Addition of other new mutations N11D and H144R can further enhance the thermophilicity of the mutant TrX. The N11D mutation may benefit the expression of the mutants.

While the present invention has described mutant xylanases which exhibit improved thermophilicity and alkalophilicity and the benefits associated with these enzymes in the production of paper pulp, these mutant xylanases may also be of use in other industrial processes, for example but not limited to the washing of precision devices and semiconductors. Further, by virtue their increased thermophilicity, and thermostability the mutant xylanses may be used in chemical processes that employ small quantities of denaturants or detergents or in the presence of solvents, for example but not limited to small amounts of apolar solvents such as but not limited to hexane, dioxanes, carbontetrachloride, benzene, ethers, chloroform, acetic acid and methylene chloride, and polar solvents such as but not limited to acetone, alcohols, dimethylformamide, acetonitrile, sulfolane, dimethylsulfoxide and water.

The present invention has been described with regard to preferred embodiments. However, it will be obvious to persons skilled in the art that a number of variations and modifications can be made without departing from the scope of the invention as described herein.

All references and citations are herein incorporated by reference

References

Arase, A., Yomo, T., Urabe, I., Hata, Y., Katsube, Y. and Okada, H. (1993) FEBS Lett. 316:123-127.

Casimir-Schenkel, J., Davis, S., Fiechter, a., Gysin, B., Murray, E., Perrolaz, J.-J. and Zimmermann, W. European Patent application no. 91810652.7, published on 04.03.92. Publication no. 0 473 545 A2.

Campbell, R. L., Rose, D. R., Sung, W. L., Yaguchi, M. and Wakarchuk, W. US patent no. 5,405,769 issued on Apr. 11, 1995.

Campbell, R. L., Rose, D. R., Sung, W. L., Yaguchi, M. and Wakarchuk, W. PCT publication no. WO 94/24270, published on 27.10.1994.

Chandra Raj, K. and Chandra, T. S. (1995) Biotechnol. Lett. 17: 309-314.

Fisk, R. S. and Simpson, C. (1993) in Stability and Stabilization of Enzymes, edited by W. J. J. van den Tweel, A. Harder and R. M. Buitelaar; published by Elsevier Science Publishers B. V. pp323-328.

Gruber, K., Klintschar, G., Hayn, M, Schlacher, A., Steiner, W. and Kratky, C. (1998) Biochemistry 37:13475-13485.

Irwin, D., Jung, E. D. and Wilson, D. B. (1994) Appl. Environ. Microbiol. 60:763-770.

Kinoshita, K., Takano, M., Koseki, T., Ito, K. and Iwano, K. (1995) J. Fermentation and Bioengineering 79:422-428.

Krengel, U. and Dijkstra (1996) J. Mol. Biol. 263:70-78.

Lee, S. L., Forsberg, C. W., and Rattray, J. B. (1987) Appl. Environ. Microbiol. 53:644-650.

Lüthi, E., Jasmat, N. B., and Bergquist, P. L. (1990) Appl. Environ. Microbiol. 56:2677-2683.

Mathrani, I. M. and Ahring, B. K. (1992) Appl. Microbiol. Biotechnol. 38:23-27.

Misset, O. (1993) in Stability and Stabilization of Enzymes, edited by W. J. J. van den Tweel, A. Harder and R. M. Buitelaar; published by Elsevier Science Publishers B. V. pp111-131.

Nissen A. M., Anker, L., Munk, N., and Lange, N. K. in Xylans and Xylanases, edited by J. Visser, G. Beldman, M. A. Kusters-van Someren and A. G. J. Voragen, published by Elsevier, Amsterdam, 1992. p325-337.

Sakka, K., Kojima Y., Kondo, T., Karita, S., Ohmiya, K. and Shimada, K. (1993) Biosci. Biotech. Biochem. 57:273-277.

Simpson, H. D., Haufler, U. R., and Daniel, R. M. (1991) Biochem. J. (1991) 277:413-417.

Sung. W. L. . Yao. F.-L., Zahab, D. M. and Narang, S. A. (1986) Proc. Natl. Acad. Sci. USA 83:561-565.

Sung, W. L., Luk, C. K., Zahab, D. M. and Wakarchuk, W. (1993) Protein Expression Purif. 4:200-206.

Sung, W. L., Luk, C. K., Chan, B., Wakarchuk, W., Yaguchi, M., Campbell, R., Willick, G., Ishikawa, K. and Zabab, D. M. (1995) Biochem. Cell. Biol. 73:253-259.

Sung, W. L., Yaguchi, M and Ishikawa, K. US patent #5,759,840, issued on Jun. 2, 1998.

Sung, W. L., Yaguchi, M and Ishikawa, K. US patent #5,866,408, issued on Feb. 2, 1999

Sunna, A. and Antranikian, G. (1997) Crit. Rev. Biotech. 17:39-67.

Tolan, J. S. and Vega Canovas, R. (1992) Pulp & Paper Canada 93:116-119).

Turenen, O., Etuaho, K., Fenel, F., Vehmaanpera, J., Wu, X. Rouvinen, J. and Leisola, M. (2001) J. Biotech. 88:37-46.

Wakarchuck W. W., Sung, W. L., Campbell, R. L., Cunningham, A., Watson, D. C. and Yaguchi, M. (1994) Protein Engineering 7:1379-1386.

Wilson, D. B., Jung, E. D., Changas, G. S., Irvin, D. C. PCT international publication on 11 may 1995. Publication No. WO 95/12668.

Winterhalter C. and Liebl, W. (1995) Appl. Environ. Bicrobiol. 61:1810-1815.

Zappe, H., Jones, W. A., and Woods, D. R. (1987) Appl.Microbiol. Biotechnol. 27:57-63.

Zappe, H., Jones, W. A., and Woods, D. R. (1990) Nucleic Acids Res. 18:2179.